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TNK2 Tyrosine Kinase as a Novel Therapeutic Target in Triple-Negative Breast Cancer

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14. ABSTRACT

Triple-negative breast cancers (TNBCs) represent only 10%-15% of all breast cancers; however, they are highly aggressive and have a higher rate of metastasis. In order to explore the role of tyrosine kinase signaling in TNBCs, we have performed global phosphotyrosine profiling for a panel of 25 TNBC cell lines. When we correlated protein phosphorylation levels with cellular oncogenic phenotypes, we observed a novel non-receptor tyrosine kinase, TNK2, to be hyperphosphorylated and activated in highly aggressive TNBC cells. Suppression of TNK2 by specific siRNAs significantly reduced the proliferation, colony formation, and invasive ability of TNBC cells. The objective of this proposal is to evaluate the therapeutic potential of TNK2 in the treatment of TNBCs. The Specific Aims of this project are: Aim 1: Do TNK2 protein levels and activation correlate with clinical and pathological features of TNBC? Aim 2: What is the value of TNK2 as a therapeutic target in vitro and in preclinical animal models? Aim 3: How is TNK2 signaling altered during oncogenesis in TNBCs?

15. SUBJECT TERMS

None listed

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INTRODUCTION:

Triple negative breast cancers (TNBC) represent 10-15% of all breast cancers. While significant advances have been made for targeted therapy of ER and HER2-positive breast cancer patients, systematic chemotherapy is the primary modality used for treatment of TNBC patients as there are no clearly defined TNBC-specific therapeutic targets (1). This proposal aims to revolutionize TNBC treatment regimens by replacing current drugs that have life-threatening toxicities with safer, more effective intervention strategies. By seeking to identify therapeutic targets in primary TNBC tumors at presentation, another overarching challenge limiting patient care that this proposal will address is the high mortality associated with metastatic breast cancer. As part of the preliminary efforts described here, we have performed a comprehensive quantitative phosphoproteomic analysis on a large panel of TNBC-derived cell lines. In this functional screen, we discovered TNK2, also known as ACK1, a non-receptor protein tyrosine kinase, was selectively activated in highly aggressive TNBC cells. In this study, we propose extending our proteomic and biochemical studies to evaluate the therapeutic potential of targeting TNK2 for inhibition in TNBCs using a novel TNK2 small molecule inhibitor, DZ1-067, also known as (R)-9b developed by the Mahajan Lab. In parallel, we will also use proteomic approaches to identify downstream signaling pathways that are specifically modulated upon TNK2 inhibition. Success of the proposed studies will lay a strong foundation to translate our preclinical discovery into a personalized clinical trial for women with TNK2 positive TNBCs and to eventually reduce the mortality rate of at least a subset of TNBC patients.

KEYWRODS: TNK2, Triple Negative Breast Cancer, Proteome, Phosphorylation, Mass Spectrometry, Signaling

ACCOMPLISHMENTS:
The major goals of the project in year 1 were as follows:

Specific Aim 1: Does TNK2 protein levels and activation correlate with clinical and pathological features of TNBC?	Timeline	Percentage of Completion	PI
Major Task 2: To evaluate TNK2 protein abundance by multiple reaction monitoring (MRM) -based mass spectrometric analysis of frozen TNBC breast cancer tissue specimens.			
Subtask 1: To select TNK2 peptides and phosphopeptide that can be detected by MRM and optimize the MS/MS	6-12	100%	Dr. Pandey

methods			
Milestone #1: Co-authors work on development of a manuscript by compiling the data together.	16-18	100%	Dr. Pandey

What was accomplished under these goals

Subtask 1: To select TNK2 peptides and phosphopeptide that can be detected by MRM and optimize the MS/MS methods

The aim of this subtask was to identify TNK2 peptides and phosphopeptides for multiple-reaction monitoring (MRM) that can be used to quantitatively assess TNK2 expression and activation in clinical breast tumor tissues. Recently, I have established a Center for Proteomic Discovery which enables us to apply most advanced mass spectrometry based technologies to our research. We have obtained two latest generation of high-resolution
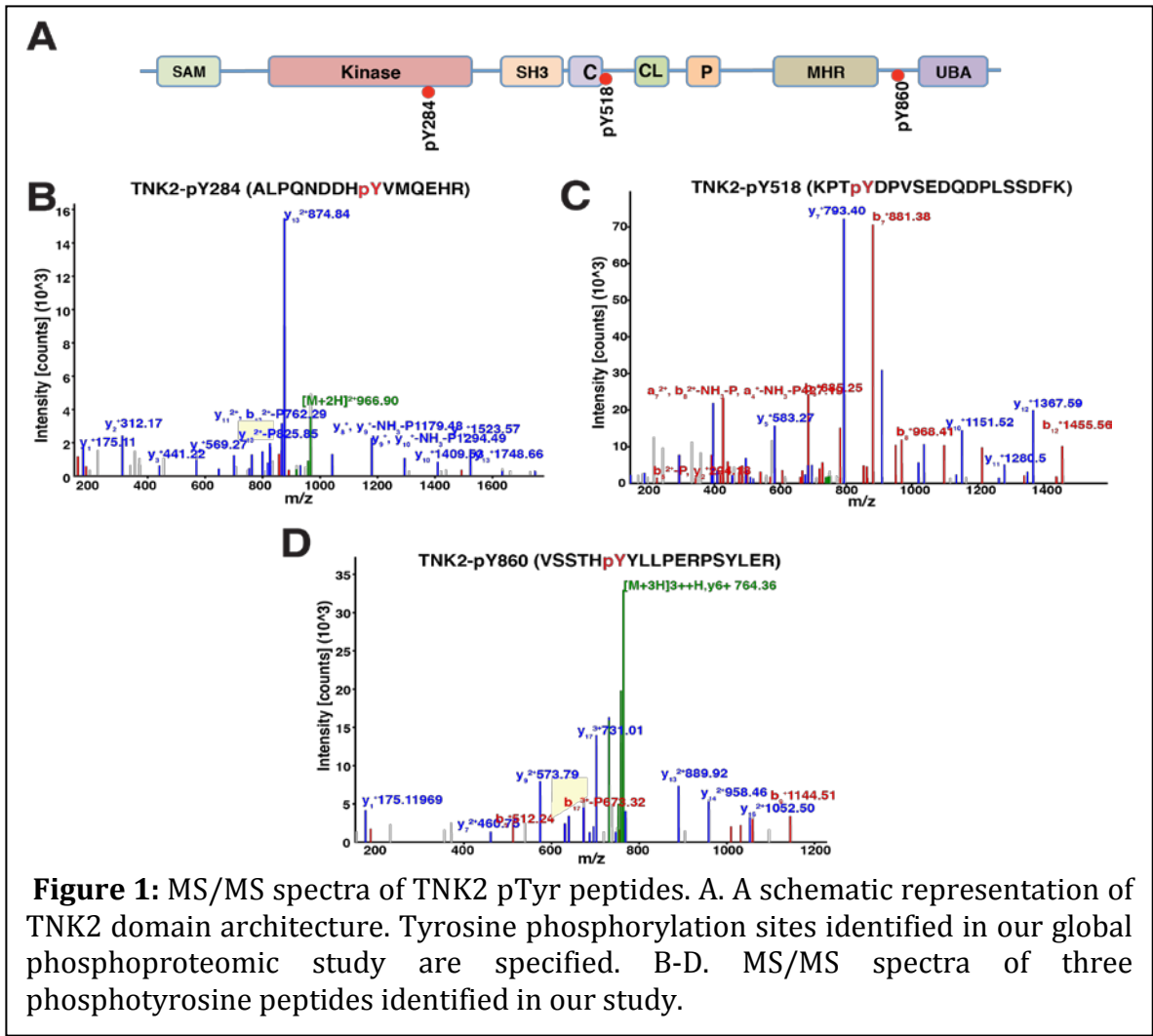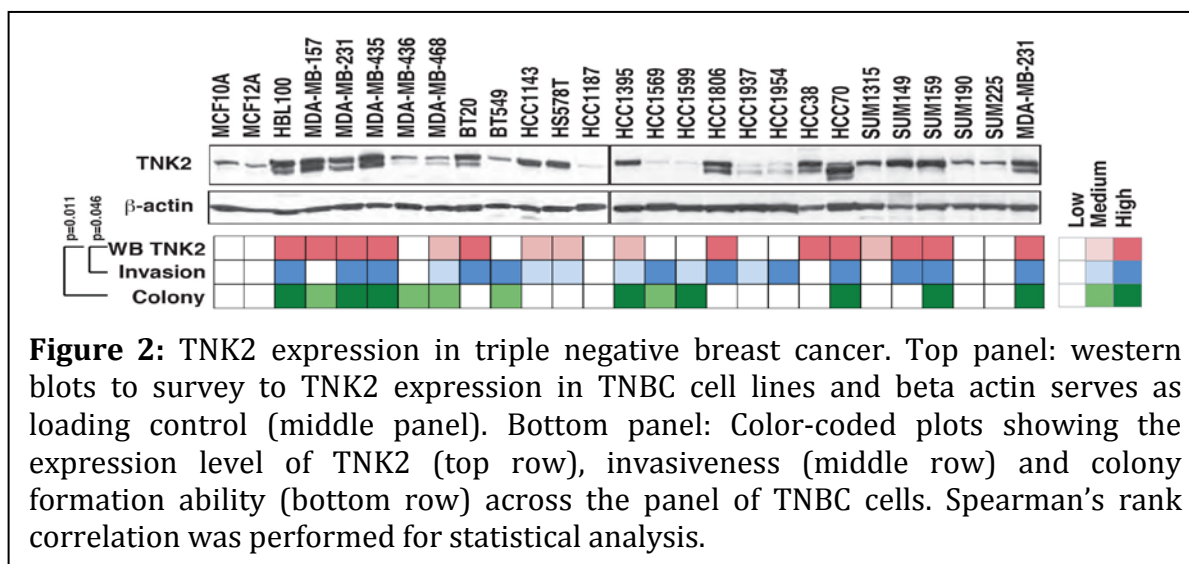

Figure 1: MS/MS spectra of TNK2 pTyr peptides. A. A schematic representation of TNK2 domain architecture. Tyrosine phosphorylation sites identified in our global phosphoproteomic study are specified. B-D. MS/MS spectra of three phosphotyrosine peptides identified in our study.

Fourier transform mass spectrometers (Orbitrap Fusion Lumos equipped with ETD)

coupled to ultra-high pressure nanoflow LC (UPLC) that enable us to perform MS analysis with the highest sensitivity possible today. With these mass spectrometers, we are able to use a new MS approach, termed parallel reaction monitoring (PRM), to develop assays to assess the TNK2 expression/phosphorylation levels. The PRM assay has emerged as an alternative method with high specificity and selectivity for targeted quantification using quadrupole-Orbitrap hybrid instruments. Comparing to conventional MRM method that can only detect a limited number of MS/MS transitions, PRM allows monitoring almost all MS/MS transitions in a very fast fashion.

In order to establish PRM assays for TNK2, we first systematically reviewed our phosphoproteomic analysis on a penal of 26 triple negative breast cancer cell lines to identify TNK2 tyrosine phosphorylated peptides that were detected in these studies. These phosphopeptides will become ideal candidates for us to develop PRM assays. Figure 1 demonstrated three phosphotyrosine peptides detected in our previously reported studies. We will develop PRM based assays to monitor the phosphorylation levels of these peptides. We plan to use several breast cancer cell lines with high expression of TNK2, as models to optimize our PRM assays for the detection TNK2. To do so, we screened TNK2 expression in the panel of 26 TNBC cell lines (Figure 2). We found that about half of the TNBC cell lines have high expression of TNK2 and the expression of TNK2 significantly associates with the invasion and colony formation ability of TNBC cells, suggesting the important role of TNK2 in tumorigenesis of TNBC. Based on these preliminary studies, we will use MDA-MB-231, HCC1395 and SUM159 as cell line models to develop and optimize PRM assays.

In the second year of this project, we will perform PRM assays for the cell line samples and clinical tumor samples.

Figure 2: TNK2 expression in triple negative breast cancer. Top panel: western blots to survey to TNK2 expression in TNBC cell lines and beta actin serves as loading control (middle panel). Bottom panel: Color-coded plots showing the expression level of TNK2 (top row), invasiveness (middle row) and colony formation ability (bottom row) across the panel of TNBC cells. Spearman's rank correlation was performed for statistical analysis.

What opportunities for training and professional development has the project provided?

This project provided an opportunity for training and professional development to Dr. Xinyan Wu. Dr. Wu is an Instructor in the Department of Biological Chemistry. Dr. Wu participated in the project and performed the mass spectrometry analysis, western blotting and cell functional assays.

How were the results disseminated to communities of interest?
Based on this study and our previous proteomics data, we published a manuscript on the journal of *Oncotarget* (See attached manuscript).

What do you plan to do during the next reporting period to accomplish the goals?
We plan to perform experiments described in Specific aim 1, Subtask 1 and 2, described briefly below:
Specific Aim 1: Does TNK2 protein levels and activation correlate with clinical and pathological features of TNBC?
Major Task 2: To evaluate TNK2 protein abundance by multiple reaction monitoring (MRM) -based mass spectrometric analysis of frozen TNBC breast cancer tissue specimens.
   Subtask 2: To perform MRM analysis with TNBC breast cancer tissue specimens.
   Subtask 3: Data acquisition and statistic analysis

Specific Aim 2: What is the value of TNK2 as a therapeutic target in vitro and in preclinical animal models?
Major Task 1: To examine oncogenic significance of TNK2 by suppressing TNK2 in TNBC cell lines.
   Subtask 1: To establish shTNK2 inducible TNBC cell lines. BT20, HS578T, HCC1806, HCC1187 and HCC1954 will be used. To assess the shTNK2 knockdown efficiency.
   Subtask 2: To suppress TNK2 expression by transient transfection of siTNK2 or induction of shTNK2 in selected TNBC cell lines. And to perform functional assays to evaluate the role of TNK2 in regulation of oncogenic phenotypes of TNBC cells.
Major Task 2: To evaluate therapeutic potential of targeting TNK2 in preclinical animal models.
   Subtask 1: To perform orthotopic xenograft with shTNK2 inducible TNBC cells and monitor tumor growth of shTNK2 induced and not induced groups. Inducible scramble shRNA (shCTLR) cells will be used as control

Specific Aim 3: How does TNK2 signaling change during the oncogenesis of TNBCs?
Major Task 1: To map signaling networks in TNBC cells with the suppression of TNK2 expression/activity.
   Subtask 1: To perform SILAC amino acid labeling for shTNK2 inducible TNBCs and

wild type TNBC cells
Subtask 2: To expand cell culture for SILAC heavy, medium and light labeled TNBC cells for phosphoproteomic analysis

IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
Breast cancer is the most prevalent cancer in women worldwide. About 15-20% of all breast cancers do not express estrogen receptor, progesterone receptor or HER2 receptor and hence are commonly classified as triple negative breast cancer (TNBC). These tumors are often relatively aggressive when compared to other types of breast cancer, and this issue is compounded by the lack of effective targeted therapy. In our phosphoproteomic profiling effort, we identified the non-receptor tyrosine kinase TNK2 as activated in a majority of aggressive TNBC cell lines. We identified three different TNK2 phosphorylation peptides in different TNBC cells (Figure 1). These TNK2 phosphopeptides will be our candidates to develop PRM assays. We have also shown that high expression of TNK2 in breast cancer cell lines correlates with high proliferation, invasion and colony forming ability (Figure 2). In collaboration with Dr. Nupam Mahajan, the partnering PI of this project, we have shown that inhibition of TNK2 with small molecule inhibitor (**R**)-9bMS significantly inhibited TNK2 kinase activity, causing loss of AKT Tyr176-phosphorylation, which compromised TNBC proliferation. Thus, our study suggests that TNK2 is a novel potential therapeutic target for the treatment of TNBC.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

CHANGES/PROBLEMS:
Nothing to Report.

Changes in approach and reasons for change
In our proposal, we planned to use multiple-reaction monitoring method to assess TNK2 expression/phosphorylation of TNK2 in breast cancer samples. We have recently equipped with latest generation of high-resolution Fourier transform mass spectrometers (Orbitrap Fusion Lumos equipped with ETD) coupled to ultra-high pressure nanoflow

LC (UPLC) that enable us to perform MS analysis with the highest sensitivity possible today. With these MS, we will use parallel reaction monitoring to assess TNK2 levels in breast cancer samples. Comparing to MRM, PRM method can generate data with higher accuracy and higher sensitivity.

Changes that had a significant impact on expenditures
Dr. Mim-Sik Kim, a postdoctoral research fellow, has left Dr. Pandey's group and joined Kyung Hee University, Korea as an Assistant Professor. Dr. Xinyan Wu will replace his role in this project. Dr. Wu is an Instructor in the Department of Biological Chemistry at Johns Hopkins University School of Medicine. The role of TNK2 in TNBC was original discovered by Dr. Wu working with Dr. Pandey (Wu et al. 2015). The participation of Dr. Wu will accelerate the progress of this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
No changes were made in use or care of vertebrate animals, or use of biohazards and/or select agents.

PRODUCTS:
Journal publications.
Authors: Xinyan Wu*, Muhammad Saddiq Zahari, Santosh Renuse, Mustafa A. Bharbuiya, Pamela L. Rojas, Dhanashree S. Kelkar, Vered Stearns, Edward Gabrielson, Pavani Malla, Saraswati Sukumar, Nupam P. Mahajan* and Akhilesh Pandey*
Title: The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer
Journal: Oncotarget
Year: 2016
Status of publication: Accepted, awaiting publication

Acknowledgement of federal support: Yes

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Nothing to Report.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?
Name: Akhilesh Pandey
Project Role: PI
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-9943-6127
Nearest person month worked: 1.2
Contribution to Project: Dr. Pandey has designed and planned experiments shown in Figure 1 and 2. Dr. Pandey also oversaw the overall progress of the project.
Funding Support:

Name: Xinyan Wu
Project Role: Instructor
Researcher Identifier (e.g. ORCID ID): 0000-0002-7745-1614
Nearest person month worked: 4
Contribution to Project: Dr. Wu has helped Dr. Pandey in planning and performing experiment shown in Figure 1.
Funding Support: Nothing to report.

What other organizations were involved as partners?
This is a Breakthrough Award, Funding Level 2 with Partnering PI, Dr. Nupam Mahajan.
The details of partnering PI and institution are as follows:
Organization Name: Moffitt Cancer Center
Location of Organization: Tampa, FL 33612, U.S.A
Partner's contribution to the project: Partnering institution has submitted their own report.
        Collaboration: Partnering institution has performed a distinct set of experiment and submitted their own report.

APPENDICES: A joint publication is attached

The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer

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ABSTRACT

Breast cancer is the most prevalent cancer in women worldwide. About 15-20% of all breast cancers do not express estrogen receptor, progesterone receptor or HER2 receptor and hence are commonly classified as triple negative breast cancer (TNBC). These tumors are often relatively aggressive when compared to other types of breast cancer, and this issue is compounded by the lack of effective targeted therapy. In our previous phosphoproteomic profiling effort, we identified the non-receptor tyrosine kinase TNK2 as activated in a majority of aggressive TNBC cell lines. In the current study, we show that high expression of TNK2 in breast cancer cell lines correlates with high proliferation, invasion and colony forming ability. We demonstrate that knockdown TNK2 expression can substantially suppress the invasiveness and proliferation advantage of TNBC cells *in vitro* and tumor formation in xenograft mouse models. Moreover, inhibition of TNK2 with small molecule inhibitor (*R*)-9bMS significantly compromised TNBC proliferation.

Finally, we find that high levels of TNK2 expression in high-grade basal-like breast cancers correlate significantly with poorer patient outcome. Taken together, our study suggests that TNK2 is a novel potential therapeutic target for the treatment of TNBC.

INTRODUCTION

Breast cancer is a heterogeneous disease, with major subtypes categorized by expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 receptor. Triple negative breast cancer (TNBC) is a subgroup of breast cancer cases that lack the expression of all these three receptors. TNBC accounts for ~15% of

invasive breast cancers and these cancers are often highly proliferative, poorly differentiated and associated with a poor prognosis [1-4]. Unlike ER positive luminal breast cancers or breast cancers with HER2 amplification that can be treated with endocrine therapy [5] or HER2-targeted therapy [6], no targeted therapy is currently available for patients with TNBCs lacking expression of ER, PR or HER2 proteins. A recent study found that several receptor

tyrosine kinases including EGFR, MET and c-Kit were transcriptionally upregulated in different subsets of TNBCs [7, 8], suggesting that these upregulated receptor tyrosine kinases in TNBCs might be potential therapeutic targets for the treatment of TNBCs. However, clinical trials of targeting receptor tyrosine kinases in TNBCs with specific kinase inhibitors have, to date, produced mainly discouraging results. For example, treatment with monoclonal anti-EGFR antibody cetuximab, alone or in combination with cytotoxic chemotherapies, showed minimal improvement in progression-free and overall survival of TNBC patients [9, 10]. These data suggest that molecules other than receptor tyrosine kinases may be involved in driving TNBCs or that one needs to further stratify the patients based on expression of individual molecular markers.

In our previous efforts to uncover the role of tyrosine kinase signaling pathways that are etiologically activated in TNBCs, we used quantitative mass spectrometry-based phosphoproteomics to globally profile the phosphotyrosine proteomes of a panel of 26 TNBC cell lines, leading to identification and quantitation of over 2,500 phosphopeptides [11]. Through systematic phenotypic characterization using invasion and soft agar colony formation assays of each cell line, we found that these TNBC cell lines were highly heterogeneous in terms of their growth rates and invasiveness. By correlating our phosphoproteomic data with oncogenic phenotypes, we identified the non-receptor tyrosine kinase, TNK2, as a protein that was hyperphosphorylated in a majority of aggressive TNBC cell lines, suggesting that TNK2 is a regulator of TNBC growth and proliferation.

TNK2, also known as ACK1 (activated Cdc42-associated kinase), is a non-receptor tyrosine kinase which has been shown to be frequently amplified or mutated in multiple human cancers including breast, esophageal, lung, ovarian, pancreatic and prostate cancer [12, 13]. Overexpression of TNK2 in cancer cell lines can increase the invasive phenotype, both *in vitro* and *in vivo*, resulting in increased mortality of mouse model of metastasis [12, 14]. Clinically, it was observed that amplification of the *TNK2* gene in primary tumors correlates with poor prognosis [12]. Further, expression of activated TNK2 was positively correlated with the severity of disease progression, and inversely correlated with the survival of breast cancer patients [15]. Being a cytoplasmic kinase, TNK2 couples extracellular signals from multiple receptor tyrosine kinases including EGFR, HER2 and PDGFR to intracellular effectors [15-18].

It has been suggested previously that phosphorylation of TNK2 might correlate with breast cancer progression [15, 19]; however, the functional significance of TNK2 expression and its role in breast cancer biology — particularly in TNBCs — has not been well elucidated. Based on emerging importance of TNK2 in various malignancies, we undertook detailed characterization of this relatively understudied non-receptor tyrosine kinase. In the current study, we show that TNK2 is overexpressed in a majority of TNBC cell lines and the TNK2 expression level is significantly associated with the aggressiveness of TNBCs. By performing genetic ablation studies, we have demonstrated that TNK2 expression not only drives TNBC proliferation but also confer invasiveness, which is reflected in tumor formation in xenograft mouse models. Further, analysis of breast cancer patient survival data revealed that high TNK2 expression is significantly correlated with worse outcome of high-grade TNBC patients. In addition, the importance of TNK2 signaling was also reflected in cell proliferation studies; treatment with TNK2-specific small molecule inhibitor, (*R*)-**9b**MS significantly suppressed growth of multiple TNBC cell lines. Taken together, these data uncover dependence of TNBCs on TNK2 signaling for both, its proliferation and invasion, and open up TNK2 inhibition as a promising therapeutic strategy for a subset of aggressive TNBCs.

RESULTS

Phenotype analyses correlate TNK2 levels with cellular aggressiveness

In our previous study characterizing the activated tyrosine signaling pathways of 26 TNBC cell lines [11], we identified four phosphotyrosine sites of TNK2 (pY284, pY518, pY859 and pY860) in multiple TNBC cell line (Figure 1A) and discovered that TNK2 pY518 is hyperphosphorylated in a majority of aggressive cell lines [11]. In order to examine the expression level of TNK2 in TNBC, we performed immunoblotting for TNK2 in our panel of 26 TNBC cell lines (Figure 1B). A significant fraction (15 out of 26) of TNBC derived cell lines exhibited medium to high levels of TNK2 expression. When we examined how TNK2 expression might be related to anchorage-independent growth and cellular invasion (as determined by colony formation and matrigel invasion assays, described in our previous study [11]), we observed that TNK2 expression levels were significantly correlated with anchorage-independent growth and cellular invasiveness, suggesting that TNK2 plays an important role in regulating oncogenicity of TNBC cells (Figure 1B). Interestingly, HBL100, one of the immortalized non-tumorigenic mammary epithelial cell lines shows a relative aggressive phenotype and high expression of TNK2 in our study. It has been shown that HBL100 cells express the oncogenic SV40 large T antigen [20]. The similar aggressive phenotype of HBL100 has also been reported by other studies [21, 22].

To further test the role of TNK2 in regulating tumorigenicity, we performed siRNA knockdown of TNK2 in four TNBC cell lines with high expression levels of TNK2 (MDA-MB-435, HCC1806, HCC70
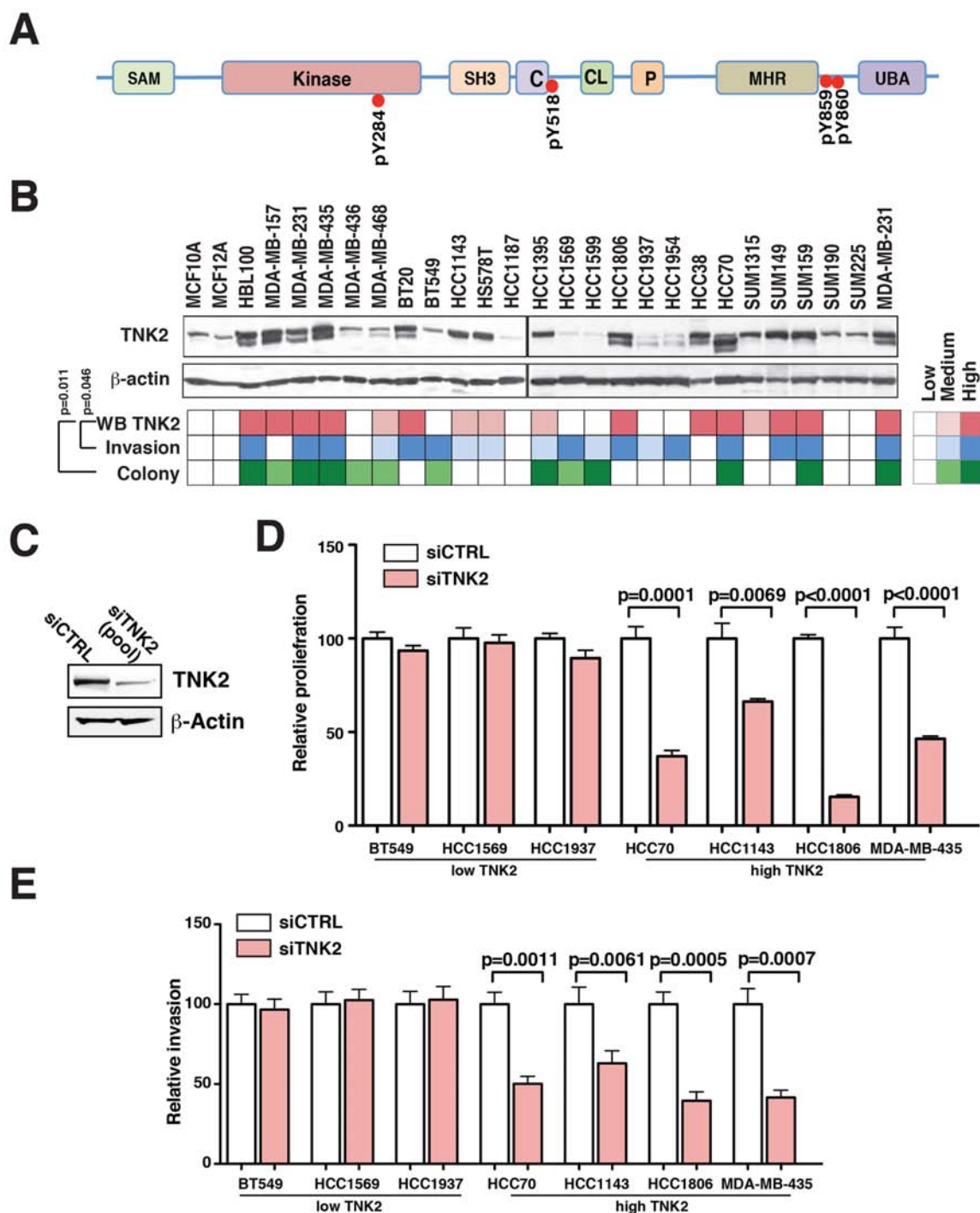
Figure 1: TNK2 is overexpressed in aggressive TNBC cell lines and required for oncogenic phenotype. A. A schematic representation of TNK2 domain architecture. Different structural domains present in TNK2 are shown. C, Cdc42-binding domain; CL, clathrin-interacting domain; kinase, tyrosine kinase domain; P, PPXY motif or WW domain-interacting region and SAM, sterile α motif. Tyrosine phosphorylation sites identified in our global phosphoproteomic study are specified. **B.** TNK2 expression level correlates with the aggressive phenotype of TNBC cells. Top panel: Western blot analysis to detect expression of TNK2 in the panel of TNBC cell lines. Color-coded plots showing the expression level of TNK2 (top row), invasiveness (middle row) and colony formation ability (bottom row) across the panel of TNBC cells. Spearman's rank correlation was performed for statistical analysis. **C.** TNK2 siRNA knockdown efficiency was examined in HCC1395 cells. 50 nM siRNA were transfected in HCC1395 cells and an immunoblot was performed to evaluate the knockdown efficiency. -Actin serves as the loading control. Proliferation **D.** and invasion assay **E.** after knockdown using siRNA against TNK2 (siTNK2) or control siRNA (siCTRL) in TNBC cells that have low expression of TNK2 (BT549, HCC1569, HCC1937) or high expression of TNK2 (HCC70, HCC1143, HCC1806, MDA-MB-435).

and HCC1143) and three cell lines with low levels of TNK2 expression (BT549, HCC1937 and HCC1569). The SMARTPool siRNA containing four different siRNA targeting different TNK2 mRNA regions was able to efficiently knockdown TNK2 expression (Figure 1C). We then performed invasion and proliferation assays on these seven manipulated cell lines and found that, in cells expressing high TNK2 levels, proliferation and invasion of cells expressing high TNK2 levels were significantly reduced by knockdown of TNK2. However, the proliferation and invasiveness of the cell lines expressing low levels of TNK2 were not affected with TNK2 knockdown (Figure 1D, 1E and Supplementary Figure S1). These results suggest that high-expression/activation of TNK2 plays an important role in regulating aggressive phenotype of TNBC cells.

TNK2 knockdown reduces aggressiveness of TNBC in vitro

Dependence of TNBCs on TNK2 signaling opens up the possibility that genetic ablation of TNK2 in these cells could suppress their proliferation and invasion. To address the role of TNK2 in aggressive breast cancers, we established a lentiviral-based Tet-ON inducible shRNA knockdown system to suppress TNK2 expression in a regulated fashion. The shTNK2 and scrambled control shRNA (shCTRL) expressing lentiviral particles were then used to infect HCC1395 and SUM159 cells—two highly aggressive TNBC cell lines with high basal expression of TNK2. Regulated induction of the shTNK2 knockdown with doxycycline was determined to be efficacious (Figure 2A, top panel). Upon induction by doxycycline, HCC1395-shTNK2 and SUM159-shTNK2 cells showed significant reduction in the ability of colony formation in soft agar (Figure 2B). To calculate cell-doubling time for HCC1395 cells with or without induction of shTNK2, we performed quantitative cell proliferation assays. As shown in Figure 2C, almost no difference in cell proliferation was observed in HCC1395-shCTRL cells regardless of doxycycline induction. However, doxycycline induction of HCC1395-shTNK2 cells substantially prolonged HCC1395 doubling time >2-fold.

Subsequently, we studied the role of TNK2 on mammosphere formation, which is commonly used to indicate cellular stemness, an important tumorigenic hallmark in breast cancer. We found that upon induction of TNK2 shRNA, the cells completely lost their ability to form mammospheres (Figure 3A). This indicates that TNK2 expression is important for these cells to maintain their stem cell-like properties. To assess the potential effects of targeting TNK2 *in vivo*, we also performed 3-D Matrigel culture assays to better mimic *in vivo* physiological conditions. Similar to our mammosphere assay results, we observed that induction of TNK2 knockdown in HCC1395-shTNK2 cells completely

eradicated colonies formed in the matrigel compared to HCC1395-shTNK2 with no induction and to HCC1395-shCTRL cells (Figure 3B), supporting to our hypothesis that TNK2 expression/activation is important for TNBC tumorigenesis *in vivo*.

We then tested whether knocking down TNK2 provided any synergistic activity with the doxorubicin treatment. We observed that cells with TNK2 knock down by shRNA became rounder and started to die at a much lower dose of doxorubicin compared to the cells that were treated with the same concentration of doxorubicin but not induced to express TNK2 shRNA, which showed a more normal morphology (Figure 3C). We performed western blots to examine the apoptosis markers including the cleavage poly(ADP-ribose) polymerase 1 (PARP) and Caspase 8. As shown in Figure 3D, the levels of cleaved PARP and Caspase 8 were substantially increased in shTNK2 knockdown cells treated with doxorubicin in comparing to the cells without shTNK2 inducstion. Taken together, these results thus suggest a synergistic effect of reducing TNK2 activity when treating TNBCs with doxorubicin.

## TNK2 knockdown reduces aggressiveness of TNBC in vivo

To test the role of TNK2 in tumor growth *in vivo,* we established orthotopic xenograft using Tet-ON shTNK2 or shCTRL HCC1395 cells transplanted into mammary fat pads of NOD-SCID mice. shRNA (shTNK2 or shCTRL) expression was induced *in vivo* by doxycycline dissolved in drinking water. As shown in Figure 4A, tumor size in the doxycycline-induced shTNK2 group was significantly reduced as compared to the control group (Figure 4A, 4B). Western blot analysis for TNK2 protein expression confirmed the decrease in TNK2 expression in doxycycline-induced shTNK2 xenograft tumors (Figure 4C). Histopathological examination of the non-induced shTNK2 tumors demonstrated a lack of well-defined capsular margins, with cells pushing the borders for invasion compared to the shTNK2-induced tumors (Figure 4D). Immunohistochemical examination of tumor sections with Ki67, a marker of proliferation, and CD31, a marker of vascular endothelial cells, revealed that the shTNK2-induced tumors indeed showed significantly lower extent of proliferation and vascularization (Figure 4D). Overall, our data shows that TNK2 plays important roles in conferring oncogenic phenotype in aggressive TNBCs.

TNK2 levels correlates with poor outcomes in patients

To address any clinical relevance of TNK2 expression, we first examined the TNK2 expression levels in TCGA dataset using Oncomine concept analysis. As shown in Figure 5A, TNK2 mRNA expression level is
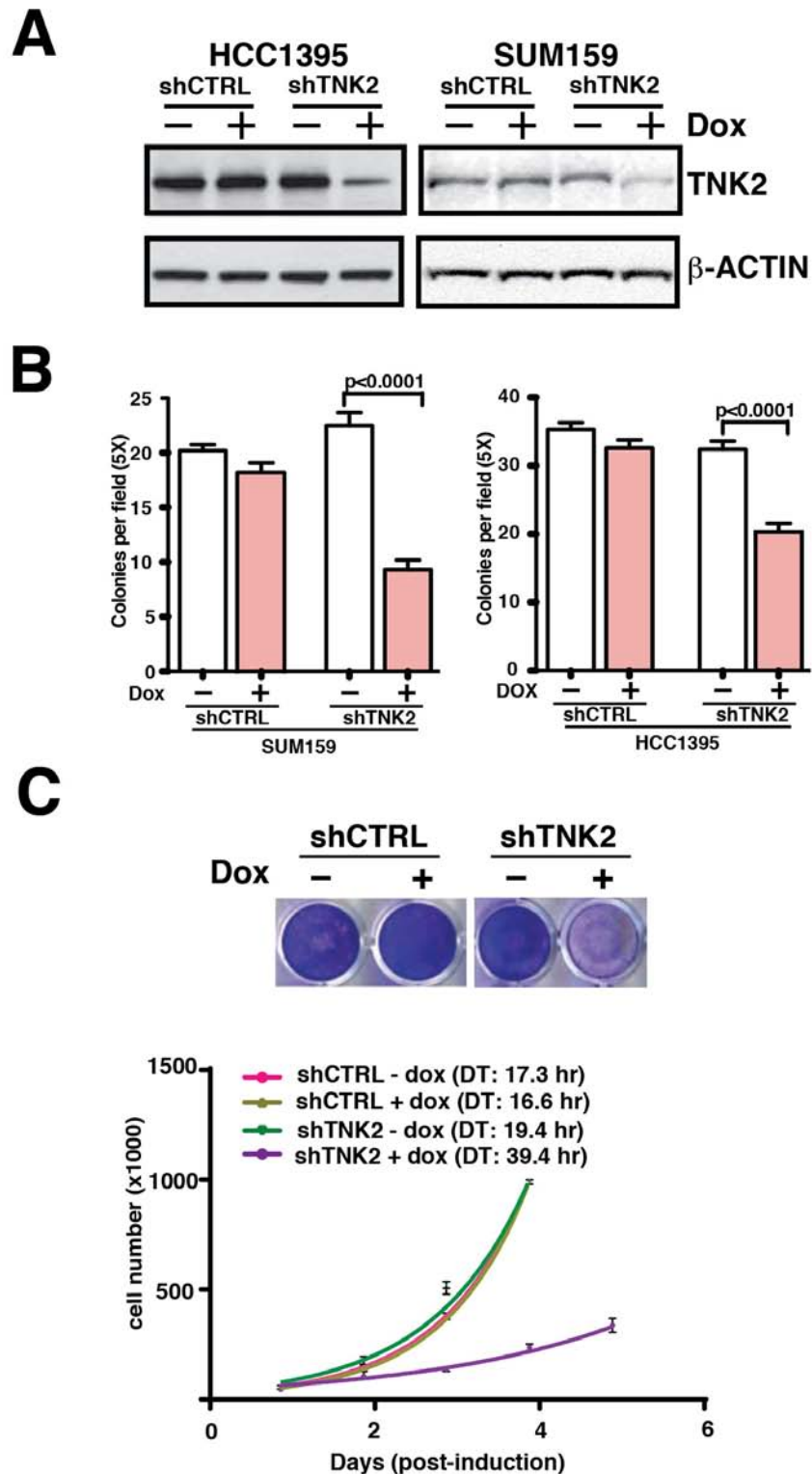
Figure 2: Inducible shRNA knockdown of TNK2 suppresses TNBC oncogenic phenotypes *in vitro*. A. Western blot analysis to examine TNK2 expression in HCC1395 and SUM159 cells infected with inducible TNK2 shRNA or scrambled control shRNA. 100 ng/ml doxycycline was used for induction. **B.** Colony formation assays with shTNK2/shCTRL transfected SUM159 and HCC1395 cells with or without doxycycline induction. Mann-Whitney tests were performed to for statistical analyses. **C.** Cell proliferation assays with shTNK2 and shCTRL HCC1395 transfected cells with or without doxycycline induction. The cells were stained with crystal violet to visualize the difference in the number of cell (top) or the doubling time calculated for each condition (bottom).
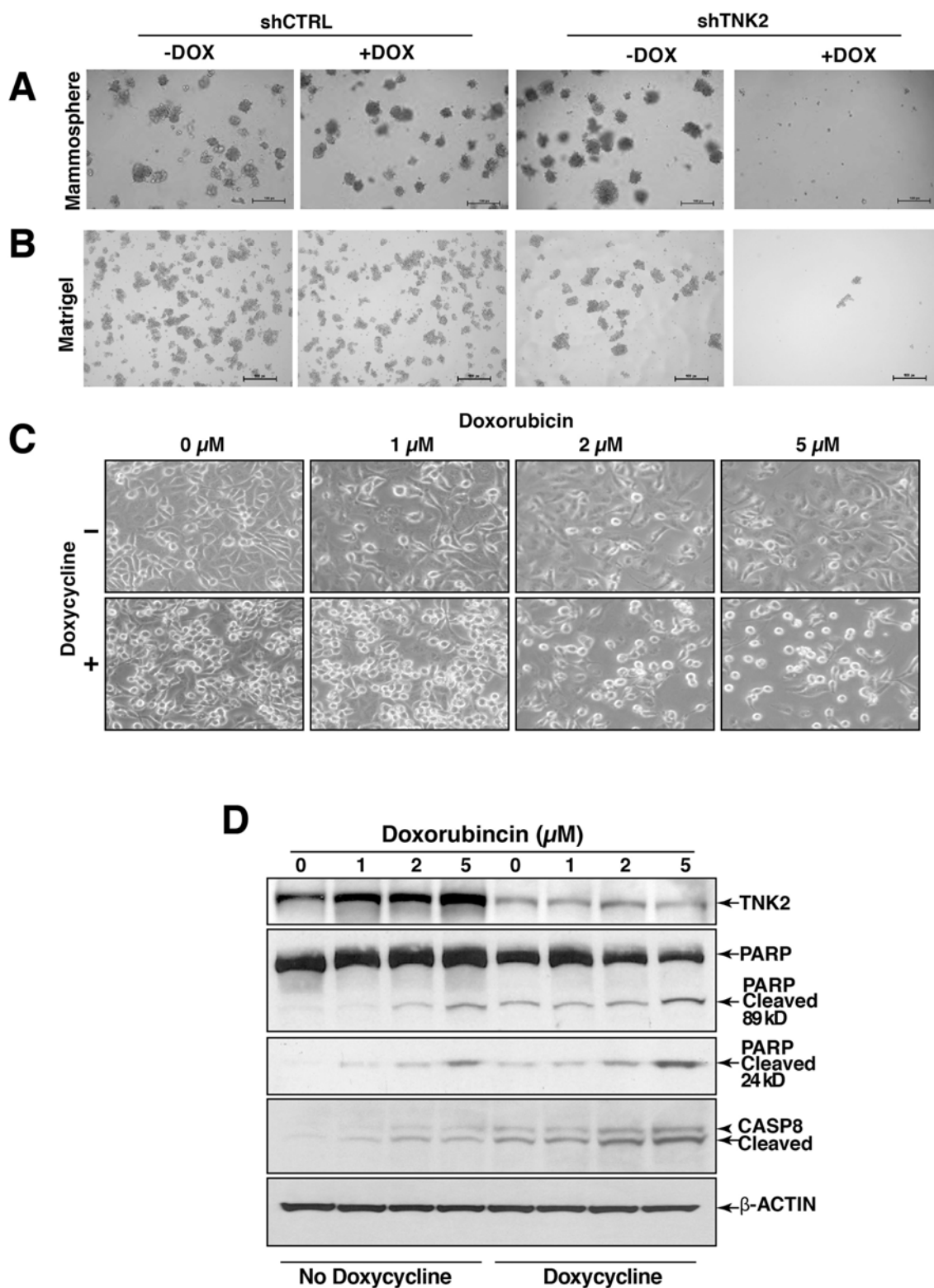
Figure 3: TNK2 is required for mammosphere formation. Mammosphere assays **A.** and 3D matrigel assays **B.** of shTNK2/shCTRL HCC1395 cells with or without doxycycline induction. **C.** Synergistic effect of knocking down TNK2 with doxorubicin treatment. shTNK2/shCTRL-infected HCC1395 cells were cultured in Petri dishes. Doxycycline was used to induce shTNK2 expression. Induced or uninduced cells were treated with different doses of doxorubicin (as indicated). **D.** Western blot analysis to examine cleaved PARP and Caspase 8 in shCTRL and shTNK2 cells treated with doxorubicin and with or without doxycycline induction of shRNA. β-Actin serves as a loading control.

lowest in normal breast and highest in TNBC comparing to other ER+ luminal and HER+ breast cancers. We also performed survival analysis using a publicly available gene expression database of breast tumors from 4,142 patients [23]. With our analysis focusing on the 669 tumors that were classified as basal-like breast cancers, we found no significant correlation between TNK2 expression and patient survival was observed, however, when a subpopulation of 237 high grade basal-like breast cancer tumors were selected for the analysis, high TNK2 expression levels were observed significantly associated with poorer patient outcomes (p<0.01) (Figure 5B). It is also worth noting that TNK2 expression did not show a statistically significant correlation with clinical outcomes for patients with high grade ER-positive tumors (Figure 5C, 5D). We believe this clinical result when added to the results from our *in vitro* and *in vivo* studies testing TNK2 inhibition on cell proliferation and tumor progression provides strong evidence to support identifying TNK2 as a novel target for personalized therapy for patients with basal-like/triple negative breast cancers that express high levels of this protein kinase.

TNK2 kinase inhibition by small molecule inhibitor suppresses TNBC proliferation

Ability of TNK2 to directly phosphorylate AKT at Tyr176 site leading to its activation [15] indicates that

TNK2-AKT signalling nexus may be involved in driving TNBC tumorigenicity. Recently, we have reported a new class of robust and soluble TNK2–specific small molecule inhibitor, (*R*)-**9b**MS [24, 25]. To address role of TNK2 in AKT Tyr176-phosphoryklation in TNBCs, cells were treated with (*R*)-**9b**MS. A significant decrease in TNK2 activation, as seen by loss of TNK2 Tyr-phosphorylation is observed upon (*R*)-**9b**MS treatment (Figure 6A). Inhibition of TNK2 activation is also reflected in significant decrease in AKT Tyr176-phosphorylation (Figure 6B). Further, several TNBC cell lines were found to be sensitive to the (*R*)-**9b**MS treatment with IC50 of 0.45 μM (MDA-MB-231), 0.48 μM (MDA MB 157), 0.6 μM (BT20), 0.67 μM (SUM159), 0.75 μM (HS578T) and 0.8 μM (HCC38) and 1.75 μM (HCC1395) (Figure 6C). Taken together, these data suggests that TNK2-AKT signaling play an important role in TNBC growth and proliferation.

DISCUSSION

In our previous study, we investigated the intrinsic heterogeneous signaling networks present within the TNBC cell population [11]. In this study, as an extension of our previous work, we focused on a non-receptor tyrosine kinase, TNK2, which is an important signaling molecule involved in integrating signaling from a number of different receptor tyrosine kinases [13]. Although TNK2 has also been reported to be amplified and mutated
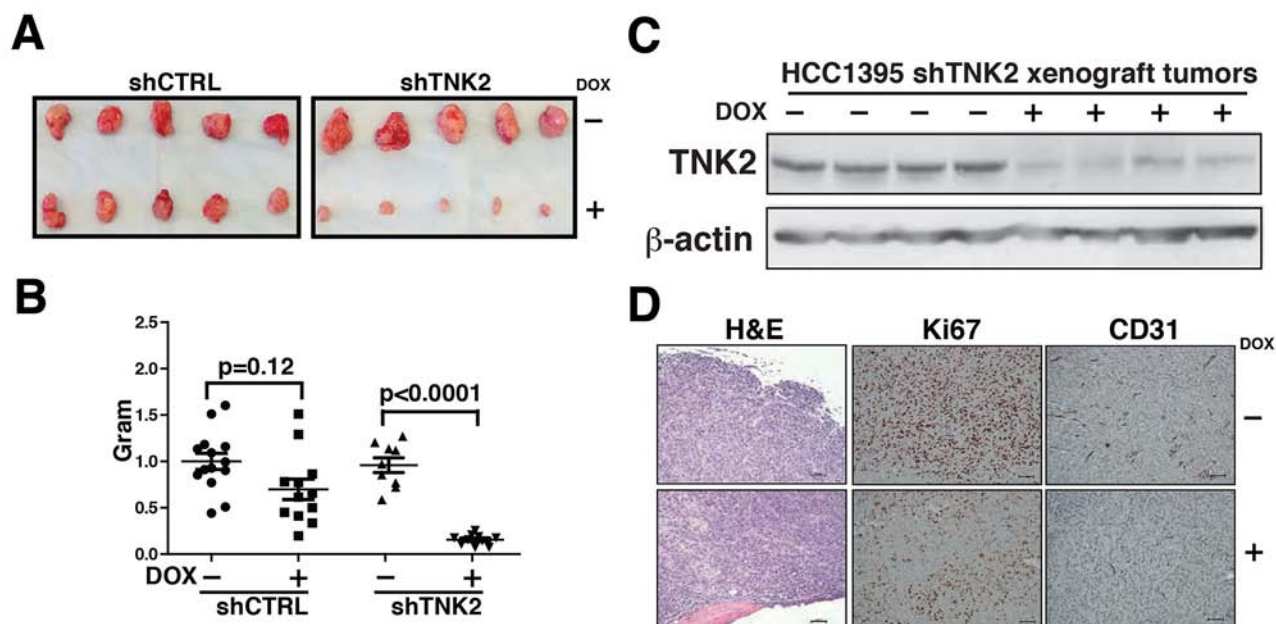

Figure 4: TNK2 knockdown suppresses tumor formation. A. Tumors harvested from mice injected with HCC1395-shTNK2 cells or HCC1395-shCTRL cells with or without doxycycline induction. **B.** Weight of tumors resected from mice xenografted with HCC1395-shCTRL or HCC1395-shTNK2 cells with or without doxycycline induction. P-values from Mann-Whitney tests to measure the statistical significance of size differences across the indicated groups are shown. **C.** Expression of TNK2 or beta actin detected by western blotting using specific antibodies in HCC1395 xenografted tumors is shown for the various tumors. **D.** Hematoxylin and eosin staining or immunohistochemical labeling for Ki67 and CD31 in HCC1395- shTNK2 xenografted tumors with (+) or without (-) induction of shTNK.

in multiple cancers [13], in breast cancer, its activation is likely to be facilitated by receptor tyrosine kinases that correlate with disease progression as well as survival.

Our global phosphotyrosine study identified several tyrosine phosphorylation sites in TNK2 (Figure 1A) in a number of TNBC cell lines and phosphorylation level of pY518 is elevated in TNBC cells with aggressive phenotypes [11]. The current study has revealed that TNK2 is not only hyperphosphorylated, but also overexpressed in many highly aggressive human TNBC tumors and also in TNBC cell lines, and that the expression of TNK2 correlates with aggressive phenotypic characteristics of TNBC cell lines. We also demonstrated

that targeting TNK2 with specific siRNA/shRNA can substantially attenuate oncogenic phenotypes of TNBC cells *in vitro* and, more importantly, dramatically reduce tumor formation in preclinical xenograft mouse models, suggesting that aggressive phenotypic characteristics are driven by TNK2. Finally, our study shows promise for combining anti-TNK2 therapy with doxorubicin treatment for these aggressive TNBCs, providing a pathway for future clinical application of our findings.

Recent studies have shown that several ubiquitin ligases including NEDD4-1 [26], NEDD4-2 [27], SIAH1 and SIAH2 [28] can ubiquitinate TNK2 and induce TNK2 degradation. Of note, in ER positive breast cancer cells,
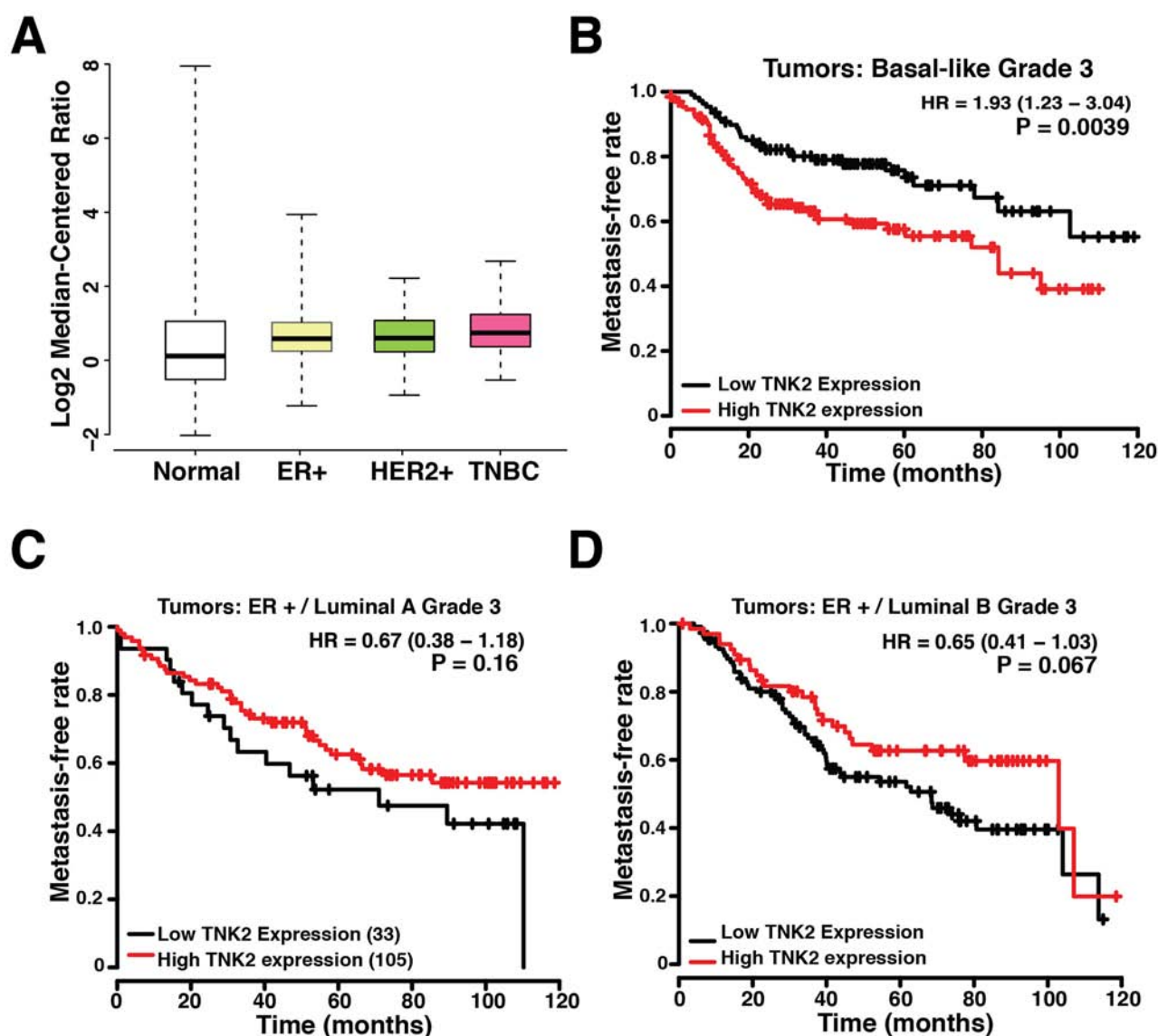
Figure 5: TNK2 levels correlate with poor outcome in patients. A. TNK2 expression level in normal breast and different subtype of breast cancers. Expression data were retrieved from Oncomine using TCGA dataset and re-plotted with R pacakge. **B.** Kaplan-Meier plot of 237 high-grade basal-like breast cancer patients stratified with high or low *TNK2* gene expression. **C-D.** Kaplan-Meier plots of 138 high-grade luminal A (C) and 184 luminal B (D) breast cancer patients. The red line represents the survival curve of patients with high expression of TNK2 and the black line represents the surviving curve of patients with low expression of TNK2.
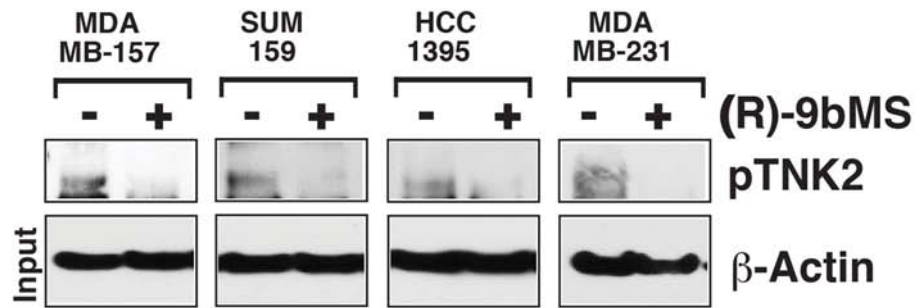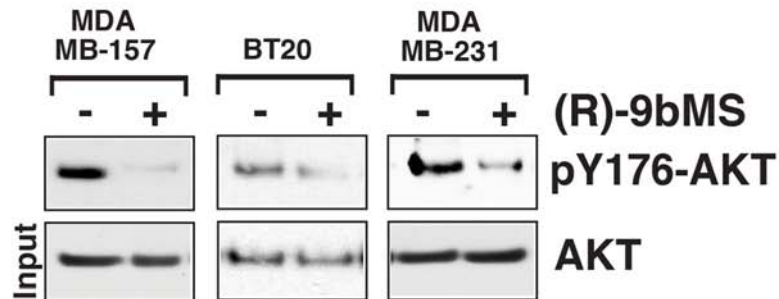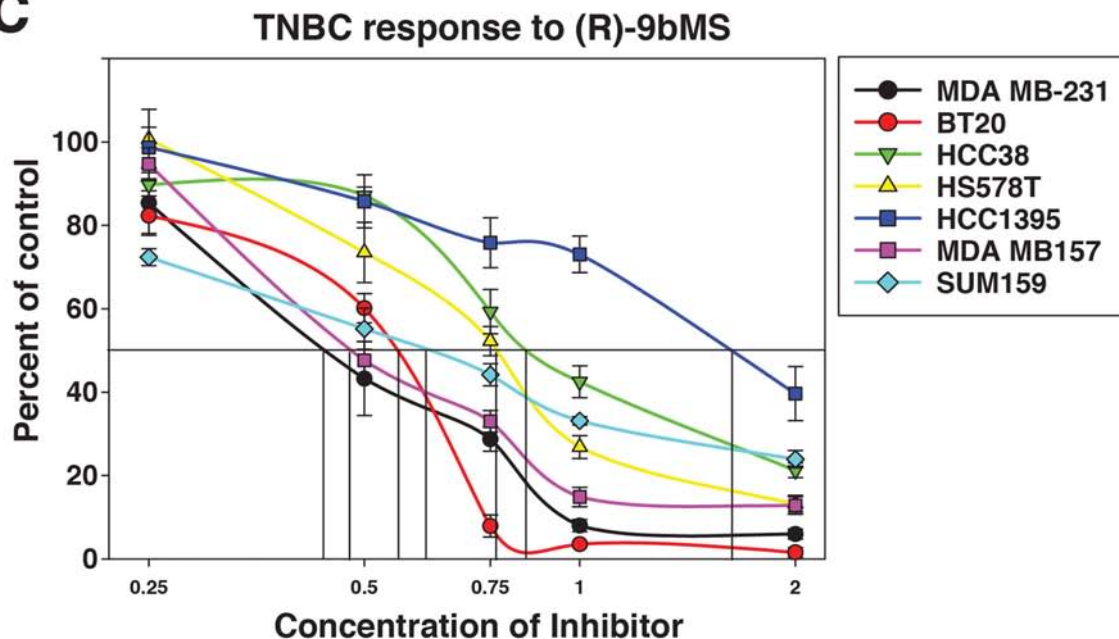
Figure 6: Small molecule inhibitor (R)-9bMS sensitizes TNK2 and its substrate AKT kinase phosphorylation compromising TNBC proliferation. A. TNBCs were treated with TNK2 inhibitor (5μM, 24 hours); lysates were immunoprecipitated with TNK2 antibodies, followed by immunoblotting with pTyr-antibodies. **B.** TNBC were treated with TNK2 inhibitor (5μM, 24 hours); lysates were immunoprecipitated with pY176-AKT antibodies, followed by immunoblotting with pan AKT-antibodies **C.** TNK2 inhibitor (*R*)-**9b**MS overcomes TNBC growth. Cells were treated with (*R*)-**9b**MS for 96hr and number of viable cells counted by trypan blue exclusion assay. Data are represented as mean ± SEM (*n* = 2, three replicates).

estrogen can activate ER and induce SIAH2 expression that subsequently ubiquitinates TNK2 and reduces TNK2 expression. Absence of ER in TNBC could be a potential reason why TNK2 is more expressed in TNBC (Figure 5A). A major downstream effector of TNK2 in hormonally regulated cancers has been AR [29], which also points to the potential of ER as another TNK2-interacting entity. However, TNBCs present a conundrum as although these cells lack expression of ER, they are exquisitely sensitive to loss of TNK2, suggesting that some other downstream effector may be operational in TNBCs. TNK2 has been shown to interact with and directly phosphorylate AKT at an evolutionarily conserved tyrosine 176 residue, promoting AKT activation. Detection of AKT Tyr176-phosphorylation in TNBCs (Figure 6A), its sensitivity to TNK2 inhibitor (R)-**9b**MS which in turn compromising proliferation of TNBCs (Figure 6B) indicates that TNK2-AKT signaling may drive TNBC tumor growth. Future studies may reveal whether sensitization of TNBCs by TNK2 inhibitor also involves other TNK2 effectors.

About 10-20% of breast cancers are found to be triple-negative. Due to limited treatment options that are currently available, there is intense interest in finding new medications that can treat this kind of breast cancer. This study not only provides evidence for 'addiction' of TNBCs to TNK2 signaling, but also provides rational for exploring TNK2-specific small molecule inhibitor (R)-**9b**MS as a realistic treatment option for TNBC patients.

MATERIALS AND METHODS

Cell culture

Cell lines were obtained from ATCC and cultured in the appropriate media as previously described [11, 30, 31].

Western blot and siRNA knockdown

Each cell line was harvested and lysed in modified RIPA, and immunoblotting was performed as previously described [32]. The primary antibodies used in this study are anti-TNK2 (Santa Cruz Biotechnology), anti-PARP cleaved 89 Kd protein (Cell Signaling), anti-PARP cleave 24 kD protein (Abcam), anti-cleaved Caspase 8 (Cell Signaling). 50 nM siRNA targeting TNK2 (L-003102, SMARTpool, Dharmacon) was used for transfections with RNAiMax (Invitrogen). Cells were harvested 48 hours post-transfection for assessing knockdown efficiency or other follow-up experiments.

Matrigel invasion assays

Cells were washed once with PBS, detached using trypsin (Life Technologies) and 5×10^4 cells were seeded into Biocoat matrigel invasion chambers (BD Biosciences). Growth media supplemented with serum for each cell line was added in the lower wells as the chemoattractant. After 24 hours, the filter membranes were stained with DAPI (Invitrogen). The number of cells that penetrated through the matrigel and membrane was counted for ten randomly selected viewing fields at 20x magnification. ImageJ was used to count cell nuclei. Student's t-test was performed for statistic analysis.

Soft agar colony formation assays

Briefly, 0.5 ml of 0.8% bottom layer agar was prepared in six-well plates. Cells with different treatment were separately trypsinized, centrifuged, resuspended in 0.4% agar medium (equal volumes of 0.8% agar and culture medium), and plated onto the top agar at 500 cells per well. The cells were grown for 14 days at 37°C. Colonies were then stained with crystal violet and counted under the microscope. Mann-Whitney tests were performed to for statistical analyses.

MTT cell proliferation assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assays were performed to measure cell proliferation. Briefly, cells that were transfected with different siRNAs in 96-well plate were left to grow for 5-7 days before the MTT assay. 1 mg/ml MTT in growth media was added into each well and the plate was incubated for two hours in 37°C. Media was then removed and 100 μl of DMSO and ethanol (1:1 by volume) was added into each well. The plate was then read for absorption at 530 nm on a microplate reader. Student's t-test was performed for statistic analysis.

To determine effect of TNK2 inhibitor, cells were treated with different concentration of (R)-**9b**MS for 96 hours and live cells (determined by trypan blue exclusion) were counted.

Generation of inducible shTNK2 cells

A shRNA construct targeting a different TNK2 sequence (GGCAGUCAGAUCCUGCAUAAG) from that targeted by siRNA was designed and cloned into the pLKO-Tet-On-puro plasmid vector (Addgene) and the sequence was confirmed by Sanger sequencing. The plasmid was then packaged into viral particles by transfection into HEK293T cells along with packaging plasmids, psPAX2 and pMD2.G. After overnight transfection, the conditioned medium was collected and used to infect SUM159 and HCC1395 cells. Stably infected cells were selected by puromycin.

In vivo tumor xenograft assays

NOD-SCID mouse xenografts were performed as previously reported [33]. Briefly, 20 four to six week old female NOD-SCID mice were purchased from NCI. HCC1395 cells stably infected with Tet-ON inducible

TNK2 shRNA or control shRNA were resuspended in matrigel:PBS (1:1 volume). $5×10^5$ cells were injected onto the mammary fat pads of NOD-SCID mice at two sites per mouse. Mice were then either administered doxycycline in drinking water (2 mg/ml) to induce the expression of shRNA or normal water as control. Five mice were used for each treatment group. After four weeks, the mice were sacrificed and tumors were processed. Student's t test was performed for the statistic analysis to compare the tumor weight. All procedures were approved by Johns Hopkins University institutional Animal Care and Use Committee and were performed in accordance with the Animal Welfare Act regulations.

Immunohistochemical labeling of xenograft tumors

Immunohistochemical staining was performed on the formalin-fixed, paraffin-embedded xenograft tumors. Briefly, paraffin embedded slides were rehydrated and then treated with hydrogen peroxide to block endogenous peroxidase activity, and then washed with PBST buffer. Slides were then blocked with goat serum before diluted rabbit polyclonal antibodies to Ki67 and CD31 were added for protein binding at 4°C and incubated overnight. The slides were washed with PBST, incubated with biotinylated secondary antibody (Dako), and treated with DAB substrate kit (Dako) according to the manufacturer's instructions. The slides were then counter-stained using Mayer's Hematoxylin (Dako) before visualizing under the microscope.

CONFLICTS OF INTEREST

N.P.M. is named as inventors on a patent application no. 60/862,763 and 62/200,084 titled 'Inhibitors of ACK1/TNK2 Tyrosine Kinase'.

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